

PATENT
Attorney Docket 4692US

NOTICE OF EXPRESS MAILING

Express Mail Mailing Label Number: EL 74053241205

Date of Deposit with USPS: January 9, 2001

Person making Deposit: Adrina Misken

APPLICATION FOR LETTERS PATENT

for

INHIBITION OF PROTEIN BINDING TO MAST CELLS

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INHIBITION OF PROTEIN BINDING TO MAST CELLS

Cross-reference to Related Applications: This application claims priority to, and is a continuation of, International Application No. PCT/NL99/00430, filed on 7 July 1999, designating the United States of America, the contents of which are incorporated by this reference, the PCT International Patent Application itself claiming priority from the Netherlands Application Serial No. 1009601 filed 9 July 1998.

Technical Field: The current invention relates to the field of immunology, and more specifically, to components which sensitize mast cells.

Background of the Invention

In the field of immunology, Askenase, P.W. et al (*J. Exp. Med.* 157, p. 862-873 (1983)) describe a “T-cell factor” which sensitizes mast cells. This T-cell factor is a very impure composition. Until now, it was not known which component/components was/were responsible for the sensitization.

Disclosure of the Invention

Applicant has found that the free light chain of immunoglobulin (Ig LC) is a constituent of the T-cell factor and is the agent responsible for the sensitization of mast cells. It has been found that Ig LC sensitizes the mast cells antigen-specifically.

Therefore the present invention relates to a compound which inhibits the binding of the free light chain of immunoglobulin to mast cells, wherein the compound, in the presence of an equimolar quantity of free light chain (LC) of immunoglobulin reduces its binding by at least 5%, said compound not being Tamm-Horsfall glycoprotein (THP), or LC-binding peptide fragments thereof.

Such a compound is of major importance for use as a means for suppressing the unpleasant effects resulting from sensitization experienced by a patient. The compounds can be detected in a simple manner, for example, by incubating a compound to be tested, together with fluorescent labeled Ig LC and mast cells. With the aid of a fluorescence microscope or, for quantitative measurement, a Fluorescence Activated Cell Sorter (FACA), inhibition may be assessed. This inhibition may occur due to competition between the compound and Ig LC for binding to the mast cell.

According to a preferred embodiment, the compound can bind to the free light chain of immunoglobulin, while the compound is capable of competing with a peptide binding to the free

light chain having the amino acid sequence (AHWSGHCCL) SEQ ID NO: 1 and that in the presence of an equimolar quantity of the peptide, the compound reduces its binding by at least 5%.

Huang Z. -Q. et al. (ref. 2) describe a unique urinary protein, Tamm-Horsefall glycoprotein (THP, also known as uromodulin) causing aggregation of immunoglobulin light chains and THP. This aggregate causes renal failure due to clogging of the distal nephron of the kidney. The publication discloses tryptic peptides of THP also causing aggregation. The use thereof or THP as a drug is not disclosed, nor is the binding thereof to mast cells disclosed.

Preferably, the compound reduces the binding of the peptide by at least 10%, preferably by at least 25%, more preferably by at least 50%, even more preferably by at least 75% and most preferably by at least 90%.

In principle, such compounds are very useful as an active component for a pharmaceutical composition, particularly if binding is reduced by more than 50%.

Within the scope of the present invention, the peptide may also be used as the active component. It is also possible to use peptides with unusual and/or modified amino acids. According to a preferred embodiment, the compound is a peptidomimeticum.

A suitable peptidomimeticum is, for example, a peptoid such as a peptoid corresponding with the peptide, but in which the side chains are located on the nitrogen atoms of the peptide backbone. In comparison with the original peptide, such a peptoid has a longer half-life in the blood. The synthesis of peptoids is well-documented in the art. The most important difference with the synthesis of peptides is the different starting materials corresponding to the amino acids.

The present invention also relates to a method of screening a series of compounds for their capability to bind the free light chain of immunoglobulin using a labeled compound capable of binding the free light chain of immunoglobulin, and capable of com-

peting with the peptide with the amino acid sequence (AHWSGHCL) of the formula sheet, wherein the screening is performed using a test comprising a competition reaction between the compound to be tested and the labelled compound. The test is suitably a homogenous test, making it possible to quickly screen compounds and to select active compounds.

In the present invention a homogenous test is understood to be a test wherein for detection it is not necessary to separate a non-complexed labelled peptide (or peptidomimeticum) from a complexed-labelled peptide. Instead of the labelled peptide with the amino acid sequence (AHWSGHCL) it is, of course, also possible to use a compound found with that peptide.

Two examples of very suitable homogenous tests are based on fluorescence (de)polarization or internal energy transfer respectively, as these allow for optimal use of respectively the difference in size between complex and labelled peptide, and the small distance between the fluorophore and chromophore.

The present invention also relates to a method of screening a series of compounds for their capability of reducing the sensitization of mast cells, wherein the screening is performed by incubating a compound to be tested and a labelled free light chain of immunoglobulin with a mast cell, and detecting reduced binding of the labelled free light chain of immunoglobulin.

In cases like this it is preferred that the screening occurs under physiological conditions, as the compound will have to be active when used as a drug under those conditions.

It goes without saying that the compound may be used for pharmaceutical purposes, especially if the compound is pharmaceutically acceptable.

Thus the present invention also relates to an application of a compound (obtained) according to the present invention or Tamm-Horsfall glycoprotein (THP) or LC-binding peptide fragments thereof for the preparation of a drug for a disease having as a symptom i) a concen-

- tration of the free light chain of immunoglobulin in serum of at least 8 mg/l, in particular of at least 15 mg/l and more in particular 20 mg/l; and/or ii) a concentration of the free light kappa-chain of immunoglobulin in spinal
- 5 fluid of at least 70 µg/l, in particular at least 100 µg/l, and more in particular 150 µg/l; and/or iii) a concentration of the free lambda-chain of immunoglobulin in spinal fluid of at least 300 µg/l, in particular at least 400 µg/l, and more in particular 500 µg/l.
- 10 Important examples of such diseases are asthma, allergy, including contact allergy and occupational allergy, chronic inflammatory bowel disorders, viral infection and multiple sclerosis. Applicant considers the possibility that migraine is also included in the list of
- 15 disorders.

According to an advantageous embodiment, a compound is used which is a peptide or peptidomimeticum with a mass of less than 10 kDaL, preferably less than 2 kDaL.

- If the peptide does not need to be synthesized
- 20 because it is derived from a protein, the mass is of less importance, although the peptide is then preferably non-immunogenic.

Thus the present invention also relates to a pharmaceutical composition comprising a compound according to

25 the invention or Tamm-Horsfall glycoprotein (THP) or LC-binding peptides thereof together with a pharmaceutically acceptable carrier or excipient.

- Finally, the invention relates to a method of diagnosing a disease in a patient having an elevated level of
- 30 the free light chain of immunoglobulin in a bodily fluid, wherein a foreign antigen specific for the disease is contacted with the bodily fluid from the patient, and subsequently the presence is determined of a complex of the foreign antigen and the free light chain of
- 35 immunoglobulin.

The bodily fluid is suitably urine, serum or plasma. Spinal fluid, lung washing and sputum are considered as well. Thus, in the context of the present invention, a bodily fluid also comprises liquids prepared

0006on the basis of the bodily material. The presence of a complex can be detected by using one of many methods known in the state of the art such as, for example, a sandwich ELISA wherein the complex is detected using a labeled antibody directed against the free light chain.

Such a labeled antibody is suitably directed against a conserved part of the free light chain.

Detailed Description of the Drawings

The present invention will now be illustrated by the following examples with reference to the drawings, wherein:

FIG. 1 represents a graph of the relative fluorescence as a measure for the amount of bound Ig LC against the number of mast cells;

FIG. 2, portions A and B, show Western blots after SDS-PAGEelectrophoresis; and

FIG. 3 shows a graph for an ELISA-based binding assay.

Detailed Description of the Invention

Preparation 1: Preparation of Ig LC-binding peptide LCBP.

The peptide Ac-AHWSGH_n-NH₂ (SEQ ID NO: 1) was prepared using a 430A Applied Biosystems Instruments (Foster City, California, U.S.A.) using solid-phase FastMoc chemistry. For the preparation a Tentagel-S-RAM resin was used as carrier material. Sensitive side chains were protected using His(Trt), Cys(TrT), Trp(boc), Ser(tBu). The peptide was released from the resin and the protective groups were removed using a mixture of trifluoroacetic acid, ethane dithiol and water (95:2.5:2.5 v/v). The raw peptide was precipitated using ether and purified by means of preparative HPLC. The purity of LCBP was verified using analytical HPLC and mass spectrometry.

Preparation 2: Isolation of lymphocyte factor

BALB/c mice (RIVM, Bilthoven, the Netherlands) were skin-sensitized using picrylchloride (PLC), dinitrofluorobenzene or oxazolone as described earlier (ref. 1). Four days after sensitization spleen cells (10×10^6 cells/ml) were cultured for 24-48 hours in RPMI medium supplemented with penicillin, streptomycin and gentamycin. The supernatant was harvested and antigen-binding proteins were isolated using hapten-affinity chromatography (bovine

gamma globulin or BSA provided with haptens immobilized to Affigel-10 (Bio Rad Labs., Veenendaal, the Netherlands)) as described by Ferguson, T.A. et al. (ref. 3). After washing the column with PBS + 0.5 M NaCl, the proteins were eluted with 5 ml 5 M guanidine solution. Subsequently, extensive dialysis against PBS took place. Proteins of biologically active samples, such as determined with an ear swelling test (see hereinafter), were fractionated using 15% Tricine SDS-PAGE, blotted onto PVDF and subsequently subjected to an Edman degradation for amino acid sequence analysis.

To determine the presence of kappa Ig LC, the haptens-binding proteins were fractionated using 12.5% SDS-PAGE, blotted onto PVDF and tested with horseradish peroxidase-labeled anti-Ig kappa LC (The Binding Site, Birmingham, U.K.) in a dilution of 1:2000. Immunoreactive proteins were visualized using ECL (Amersham Pharmacia Biotech Benelux, Roosendaal, the Netherlands) according to the manufacturer's recommendations (FIG. 2, portion A). This showed that in lymphocyte factors specific for picric acid, dinitrofluorobenzene and exazolon respectively, the presence of Ic LC could be demonstrated using an anti-kappa Ig LC-specific antibody.

FIG. 2, portion B shows that lymphocyte factor comprises a large variety of antigen-binding proteins. The lanes labeled A (eluted with 0.2 MN Na₂CO₃) and B (void volume of column) are two fractions obtained using affinity chromatography. Of these fractions only fraction A exhibited the biological activity demonstrated in Example 3. Of the protein with an apparent molecular weight of 27 kDa (p27) (DIQMTQSPPSLSAXLG)(SEQ ID NO: 2) the N-terminal amino acid sequence was determined, which corresponded to the sequence of Ig LC (DIQMTQSPSSLSASLG)(SEQ ID NO: 3) known from the literature.

Example 1: Ig LC-binding to mast cells

Basophilic leukemia cells RBL-2H3 (a gift of C. Fewtrell, Ithaca, NY, U.S.A.) of the rat, an established model for mast cells, were incubated with 200 ng/10⁵ cells Ig LC labeled with fluorescein isothiocyanate. They were incubated for 30 minutes at 4°C in the presence or absence

of 250 µg/ml of the peptide LCBP prepared in preparation 1 (peptide binding to the light chain). Subsequently they were washed using a phosphate-buffered saline supplemented with 1% v/v foetal calf serum and 0.01% w/v sodium azide.

- 5 Binding of FITC-labelled Ig LC to RBL-2H3 cells was analyzed using a FACScan flow cytometer.

The curve 1 of Fig. 1 shows that the free light chain of Ig binds to mast cells. Secondly, curves 2 and 3 of Fig. 1 show that this binding can be inhibited using 10 LCBP (0.25 mg/ml and 0.50 mg/ml respectively). Curve 4 represents the autofluorescence of unlabelled RBL-2H3 cells.

Example 2: Effect of peptide of LCBP to the airduct response

15 2.1 Sensitization of mice

Lightly anaesthetized with halothane, mice were passively sensitized by injection with trinitrophenyl (TNP)-specific Ig LC (2 µg in 50 µl of sterile saline) in the retroorbital plexus. Control mice received only 50 µl 20 of sterile saline. Thirty min. after injection, while being lightly anaesthetized with halothane, all mice received intranasally 50 µl PSA-solution (picrylsulphonic acid dissolved in phosphate-buffered saline).

2.2 Effect of Ig LC and LCBP

25 A part of each of these two groups of mice simultaneously received 200 µg LCBP (the peptide prepared in Example 1) intranasally.

Bronchoconstriction was measured as described by Kraneveld A.D. et al. (ref. 3) and Zuany-Amorim et al. 30 (ref. 6). In short, 5 minutes before intranasal application of PSA, mice were placed in a plethysmographic chamber (Buxco Electronics Inc., Shanon, CT) in order to analyze respiration and to obtain basal line readings. After the intranasal administration the animals were directly 35 returned to the chamber. The respiratory resistance was measured for a period of 45 minutes. The respiratory resistance is expressed as a dimensionless value calculated by using the formula for the Penh (ref. 4). For each

mouse the maximum Penh values were measured during an interval of 1 minute at the moments shown in Table 1.

TABLE 1

time (min.)	PBS/PBS/PSA (Penh)	PBS/Ig LC/PSA (Penh)	LCBP/PBS/PSA (Penh)	LCBP/Ig LC/PSA (Penh)
5	0 ¹	0,62±0,09	0,35±0,03	0,44±0,05
	2,5	0,60±0,20	1,98±0,16	0,60±0,12
	5	0,62±0,20	2,29±0,51	0,65±0,23
	7,5	0,70±0,30	5,29±1,00	0,74±0,12
	10	0,60±0,20	6,05±1,90	0,53±0,03
	15	0,70±0,05	3,76±0,70	0,49±0,06
10	20	0,73±0,20	1,88±0,30	0,49±0,03
				0,52±0,05

15 ¹ Basal line reading before the challenge

This experiment shows that intranasal administration of LCBP during the passive sensitization (i.v.) with Ig LC can completely inhibit the bronchoconstriction (elevation 20 of Penh) induced by antigen (PSA).

Example 3: Effect of passive sensitization with Ig LC on ear swelling.

Mice were, as described by Example 2.1, passively sensitized by injection with a lymphocyte factor PLC-F 25 obtained from a mouse sensitized with picrylchloride or Ig LC specific for trinitrophenyl. Control mice received either only PBS or TNP-specific Ig HC (heavy chain of immunoglobulin). Thirty minutes after injection picrylchloride (50 µl 0.8% picrylchloride (PCL) dissolved 30 in olive oil) was applied to the ear. After 2 hours, the thickness of the ear was measured (Table 2).

TABLE 2

5	Treatment	Increase in ear thickness (x 10 ⁻⁵ m)
	PCL-F	3,94 ± 0,56
	TNP-specific Ig LC	3,77 ± 0,46
	PBS*	0,37 ± 0,40
10	TNP-specific Ig HC*	0,01 ± 0,32

* Control

This experiment shows that TNP-specific Ig LC has the same effect as lymphocyte factor. The heavy chain does not show
15 this effect.

Example 4: Bronchoconstriction with mast-cell deficient mice

Mast cell-deficient mice (WBB6F1 W/Wv) (Jackson Labs, Bar Harbor, ME, USA belong to a strain of mice lacking mast cells. Their response to challenge with picryl sulphonic acid 30 minutes after sensitisation with PBS (vehicle), LC and IgE (5 microgram in 50 microliter each) was compared with the response of strain WBB6F1 +/- (Jackson Labs, Bar Harbor, ME, USA) mice having a similar genetic make-up but not mast-cell deficient. Picryl sulphonic acid (50 microliter, 0,6% (w/v)) was administered intranasaly and the Penh values were obtained as described in Example 2. The results are given in Table 3.

TABLE 3

Time (min.)	PBS	LC	IgE
Mast cell-deficient animals			
5	mean	mean	mean
0	0,88 (0,11)	0,72 (0,10)	0,84 (0,10)
2,5	1,15 (0,44)	0,82 (0,23)	1,84 (0,60)
5	1,15 (0,17)	1,01 (0,16)	1,96 (0,48)
10	1,37 (0,35)	1,24 (0,41)	2,51 (0,68)
15	1,22 (0,36)	1,07 (0,18)	2,02 (0,89)
20	0,82 (0,12)	0,85 (0,19)	1,21 (0,26)
control			
15	0,72 (0,04)	0,98 (0,12)	0,95 (0,21)
2,5	1,02 (0,22)	2,46 (0,51)	2,17 (0,44)
5	1,25 (0,22)	6,14 (1,87)	4,34 (1,97)
7,5	1,05 (0,23)	8,17 (1,10)	4,73 (1,32)
10	1,33 (0,15)	8,89 (1,95)	6,83 (1,22)
20	1,17 (0,19)	4,41 (1,30)	3,97 (0,86)
	0,98 (0,20)	2,58 (0,65)	2,04 (0,43)

The standard error (SE) is shown between parenthesis

From this table it can be seen that the mast-cell
25 deficient mice are not sensitized by LC.

Example 5: ELISA-based binding assay

Wells of a microtiter plate were coated at room temperature overnight with 2 µg/ml immunoglobulin light chains or, as a first control, Bovine Serum Albumin (BSA).
30 Also, as a second control, wells were treated with 250 mM glycine buffer, pH=9.5. The wells were emptied and washed 5 times with 0,05% Tween-20 in PBS. The wells were blocked with HPE-buffer (High Performance Elisa buffer, CLB, Amsterdam, The Netherlands) for 1 hour, and subsequently 35 the wells were washed again with 0,05% Tween-20 in PBS. Human uromodulin was diluted in HPE-buffer and incubated for 2 hours. The wells were washed 5 times with 0,05% Tween-20 in PBS.

To detect bound uromodulin, 1/5000 diluted rabbit anti-human uromodulin antiserum was added (Anawa Trading, Zürich, Switzerland) and incubated for 1 hour. After washing 5 times with PBS, 0.05% Tween20 anti-rabbit-IgG conjugated to horse radish peroxidase (CLB) was added and incubated for 1 hour. Bound peroxidase was detected as is well-known in the art using 3,5,3',5'-tetramethylbenzidine/H₂O₂ in 0.11 M sodium acetate pH 5.5. The reaction was stopped using an equal volume of 2 M H₂SO₄ and the absorbance was read at 450 nm. The data obtained are depicted in Fig. 3 which shows that a uromodulin concentration within, for example, 4 - 40 µg/ml, is an excellent concentration for repeating the above ELISA to detect novel compounds according to the present invention. To this end, uromodulin and the compound (preferably at several concentrations) to be investigated are incubated simultaneously in order to compete with each other.